American Journal of Hematology 54:242–248 (1997)

# **Ankyrin Bugey:** A De Novo Deletional Frameshift Variant in Exon 6 of the Ankyrin Gene Associated With Spherocytosis

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**We describe a case of spherocytosis in a French child splenectomized at age 10 years. The parents were devoid of any clinical, hematological, or biochemical abnormalities. Following splenectomy, the proposita exhibited a reduction of red cell membrane ankyrin. The variable number of dinucleotide repeats associated with the erythroid ankyrin gene (*ANK1*) were studied at the genomic level. The father, the mother, and the proposita had the $AC_{14}/AC_{11}$, $AC_{14}/AC_{14}$, and $AC_{14}/AC_{11}$ genotypes, respectively, although the proposita exhibited a pattern consistent with an $AC_{14}$,-combination at the cDNA level. We thought there could be a de novo mutation in the *ANK1* allele of paternal origin ($AC_{11}$). A false paternity seemed most unlikely. Based on PCR-amplification of exons, SSCP analysis, and, when appropriate, nucleotide sequencing, we found a one-nucleotide deletion in codon 146 (exon 6): 521delC, ACG→AG. This placed in phase a TAG triplet normally overlapping codons 150 and 151. Early interruption of translation presumably accounted for the premature degradation of mutant mRNA. Restriction analysis confirmed the presence of the mutation in the proposita and its absence in the parents. The variant was designated ankyrin Bugey. Am. J. Hematol. 54:242–248, 1997** © 1997 Wiley-Liss, Inc.

**Key words: hereditary spherocytosis; ankyrin mutation; ankyrin deficiency; de novo mutation**

## INTRODUCTION

Hereditary spherocytosis (HS) is the most common congenital hemolytic anemia due to membrane protein defects. A subset of HS cases stems from alterations to the gene (*ANK1*) encoding erythroid ankyrin (reviewed in Lux and Palek [1]). Attention was initially drawn to a deficiency of spectrin [2,3], but this highly significant alteration turned out to be secondary in a number of patients with dominant HS. On the other hand, the possibility that the *ANK1* gene could carry the primary alteration became more and more likely on the basis of cytogenetic abnormalities [4–7], decrease in ankyrin content [7–12] and, when investigated, reduction in the synthesis of ankyrin [13], linkage of HS [14] with an intragenic *Nco*I polymorphic site of the *ANK1* gene [15], and occurrence of truncated ankyrin variants [16,17].

Recently, Eber et al. [18] and Miraglia del Giudice et al. [19] found highly heterogeneous mutations while screening the exons of the ankyrin gene using PCR, single-strand conformational polymorphism (SSCP) analysis [20], and nucleotide sequencing of the polymorphic exons. Dominantly inherited cases of HS were usually associated with frameshift or nonsense mutations [18]. Other authors established that these kinds of mutations lead to the absence of mRNA from one *ANK1* allele in 20% of HS cases [21]. Recessively inherited cases were associated with changes, including single amino-acid substitutions or a single base substitution in the promoter region [18].

We report a new variant, ankyrin Bugey detected using

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Received 25 April 1996; Accepted 10 October 1996.

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**TABLE I. Routine Red-Cell Parameters***

| | RBC ($10^{12}$/l) | HB (g/l) | MCV (fl) | Reticulocytes ($10^9$/l) | Morphology | Osmotic resistance (pink test) (%)[a] |
|---|---|---|---|---|---|---|
| I.1 | 5.61 | 165 | 90 | nd | Normal | 5.1 |
| I.2 | 4.83 | 141 | 90 | nd | Normal | 6.3 |
| II.1 | 4.57 | 132 | 87 | nd | Normal | 8.2 |
| II.2 | 5.09 | 138 | 82 | nd | Normal | 4.0 |
| II.3[b] | 4.24 | 108 | 79 | 242 | Presence of spherocytes | 48.3 |
| II.3[c] | 5.39 | 146 | 78 | 45 | Presence of spherocytes | 19.1 |
| II.4 | 5.15 | 131 | 82 | nd | Normal | 4.3 |

*I.1, father; I.2, mother; II.1–II.4, children; II.3, proband.

[a]Osmotic resistance was determined after 24 hr based on the pink test [23]; normal values, <16%. ND, not determined.

[b,c]Before and after splenectomy, respectively.

SSCP analysis. The picture was that of a recessively inherited condition. However, differences in the variable number of dinucleotide repeat $AC_n$ [22], between the genomic DNA and the cDNA, suggested that the *ANK1* allele of paternal origin had been inactivated by a de novo mutation, accounting for the absence of nearly complete absence of the corresponding mRNA. The de novo mutation was a single base deletion in codon 146 (exon 6): 521delC, A<u>C</u>G→AG, resulting in an in-frame stop codon a short distance downstream.

## CASE REPORT

Informed consent was obtained from the patients, and all procedures were performed according to the Declaration of Helsinki. Family NI is of French (father)-Italian (mother) ancestry (Table I). The proposita (child II.3) was born in 1982. Pallor was reported in early life. The first thorough hematological examination was performed in 1987. Child II.3 appeared pale but nonicteric. The spleen was palpable 3 cm below the costal margin. Routine laboratory tests showed a mild hemolytic anemia (Table I). Spherocytes (10%) were visible on smears. A systematic study of the family showed that the parents and the other children had no clinical or hematological abnormalities (Table I). In 1988, child II.3 suffered an aplastic crisis, with reticulocytopenia (12,000/$mm^3$) followed by a reticulocytosic crisis (534,000/$mm^3$). One transfusion was required. No parvoviral infection was detected by serological tests. The pink test [23] showed an increased osmotic fragility for proposita II.3, while this test was normal for all other family members (Table I). The proposita underwent splenectomy in 1992 (spleen, 500 g), and major clinical improvement ensued (1993) (Table I). The clinical picture was consistent with that of a recessive inherited HS.

Because this study suggested the presence of a de novo mutation in the proposita, bearing on the paternal allele (see below), paternity tests were carried out in a specialized laboratory according to Monson et al. [24]. The results did not exclude paternity, with an error risk close to 1/13,500,000.

## METHODS

### Protein Analysis

Analysis of red cell membrane proteins was carried out using polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (SDS-PAGE) according to Fairbanks et al. [25] and Laemmli [26], as previously described [19]. The amounts of pertinent bands were expressed as percentages of band 3. In this report, we focused on ankyrin bands 2.1 and 2.2. It is known that a high reticulocyte count, as was encountered before splenectomy, increase the proportions of ankyrin isoforms 2.1 and 2.2, and consequently is liable to mask a reduction of these components. Measurements of bands 2.3 and 2.6 was not accurate enough to allow them to be taken into account.

### cDNA Analysis

Total reticulocyte mRNA extraction, reverse transcription (RT), and amplification using the polymerase chain reaction (PCR) were performed as previously described [27]. PCR primers A (sense, $^{6276}$TCCCAGATCGCTCTACATGA) and B (antisense, $^{6385}$CACAGCTTCAGAAGTCACAG) [22] were used to screen the variable number of dinucleotide repeats (VNDR) associated with the *ANK1* gene. Twenty-five PCR cycles were carried out under the following conditions for denaturation, annealing, and extension, respectively: 92°C, 1 min; 58°C, 30 sec; and 72°C, 1 min. The fragments obtained were separated by electrophoresis using a 12% polyacrylamide gel followed by silver staining, or submitted to direct

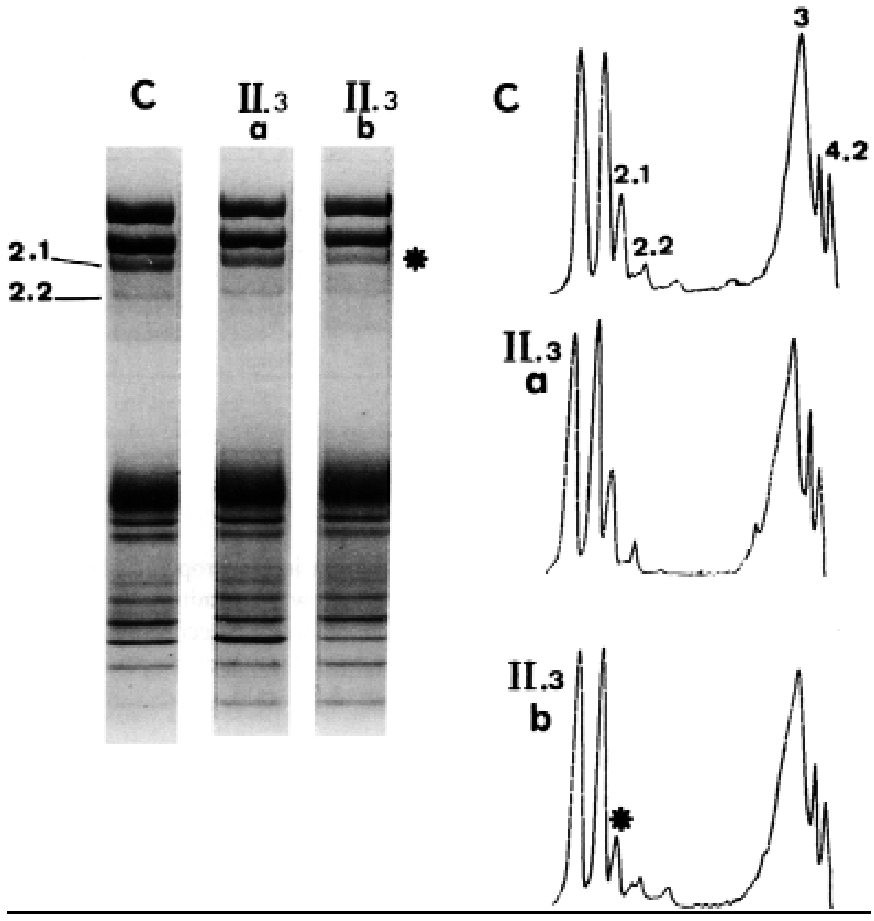


**Fig. 1. SDS-PAGE analysis of red blood cell membrane proteins. C, control. II.3, proposita prior to (a) and following (b) splenectomy. Densitometric scans of stained gels are shown at right, and quantitative results are presented in Table II. *Reduction of ankyrin band 2.1 following splenectomy.**

nucleotide sequencing. RT-PCR was performed to study the expression level of the mutated mRNA.

The PCR step (primer C: sense, $^{290}$TCATTCTAGAAACGACAACCA; primer D: antisense, $^{731}$TAGTGAGCCGCAATGTGCA) was performed as follows (30 cycles): 92°C, 1 min; 56°C, 1 min; and 72°C, 1 min. Larger cDNA fragments were amplified using primers C and F (antisense, $^{1423}$CTCTGGCTGCCATGTGTAG) and annealing at 58°C. Formamide (4%) was present in the medium for all PCR steps. PCR products were digested with *Alw*NI and analyzed on Nusieve 3% agarose gels (FMC Bioproducts, Rockland, ME).

## Genomic DNA Analysis

Genomic DNA was obtained from leukocytes as previously described [28]. Exons 1–6 were PCR-amplified as follows (30 cycles): 92°C, 1 min; 1 min at the appropriate temperature for hybridization; and 72°C, 1 min, using sets of intronic primers designed from genomic sequences (B.G. Forget, unpublished data). Primers used for exon 6 amplification were as follows: primer F (sense, $^{-47}$tggcgtcagacgagtcaga), primer G (antisense, $^{+85}$ttctcctgcctgggaagtc), annealing temperature, 58°C. The resulting fragments were subjected to SSCP analysis [20]. The PCR-amplified fragments carrying exon 6 were cloned using the TA cloning kit (Invitrogen, San Diego, CA). Nucleotide sequencing was performed using the dideoxynucleotide technique, with universal primers and dITP. After the termination reaction, terminal deoxynucleotidyl transferase was added to improve the sequence pattern [29].

The *ANK1* gene *Nco*I polymorphism lying in the 3′ region was analyzed as described by Gallagher et al. [15]. PCR analysis of the variable number of dinucleotide repeats (VNDR) associated with the *ANK1* gene was performed using primers A and B [22] (25 cycles: 92°C, 1 min; 58°C, 1 min; and 72°C, 1 min) followed by electrophoresis as described above. The mutation was screened by PCR amplification of a genomic DNA segment encompassing exon 6 (primers F and G; 30 cycles: 92°C, 1 min; 58°C, 1 min; and 72°C, 1 min; 4% formamide) followed by *Alw*NI digestion.

**TABLE II. Quantification of Pertinent Membrane Proteins***

| Bands | I.1 | I.2 | II.3 Presplenectomy | II.3 Postsplenectomy | Controls (n = 5) |
|---|---|---|---|---|---|
| 2.1 | 14.6 | 16.5 | 17.0 ± 0.8 | 9.9 ± 0.4 | 14.9 ± 1.1 |
| 2.2 | 5.0 | 5.1 | 4.7 ± 0.1 | 3.7 ± 0.1 | 5.4 ± 0.4 |
| Sum | 19.6 | 21.6 | 21.6 ± 0.9 | 13.6 ± 0.5 (−33%) | 20.3 ± 1.5 |
| Spectrin (α + β) | 98.0 | 101.2 | 85.4 ± 3.2 (−11%) | 85.7 ± 3.4 (−11%) | 96.1 ± 5.2 |
| Protein 4.2 | 14.3 | 14.2 | 12.6 (−9%) | 13.0 (−5.8%) | 13.8 ± 0.4 |

*Coomassie-blue stained bands on SDS-PAGE gels were scanned (570 nm). Surface areas of the bands were expressed as percentages of band 3 peak surface. Spectrin and ankyrin were quantified according to Fairbanks *et al.* [25], whereas protein 4.2 amount was evaluated from the gel of Laemmli [26]. In child II.3, values for bands 2.1, 2.2, and spectrin represented the mean value of three determinations. Variations are indicated in brackets when significant ($P < 0.05$).

**TABLE III. *Nco*I Polymorphic Site and VNDR Analysis***

| | *Nco*I | $(AC)_n$ |
|---|---|---|
| cDNA | | |
| I.1 | | $AC_{14}$, $AC_{11}$ |
| II.3 | | $AC_{14}$,− |
| Genomic DNA | | |
| I.1 | +/− | $AC_{14}/AC_{11}$ |
| I.2 | +/+ | $AC_{14}/AC_{14}$ |
| II.3 | +/+ | $AC_{14}/AC_{11}$ |

*Intronic *Nco*I polymorphic site was analyzed by PCR. The products were digested with *Nco*I and monitored using 1% agarose gel electrophoresis. Concerning the (AC)n VNDR, genomic and complementary cDNA were PCR-amplified using primers A and B (See text). The fragments obtained were resolved using 12% polyacrylamide gel and silver stained. The number of (AC) repeats (11 or 14) are indicated.

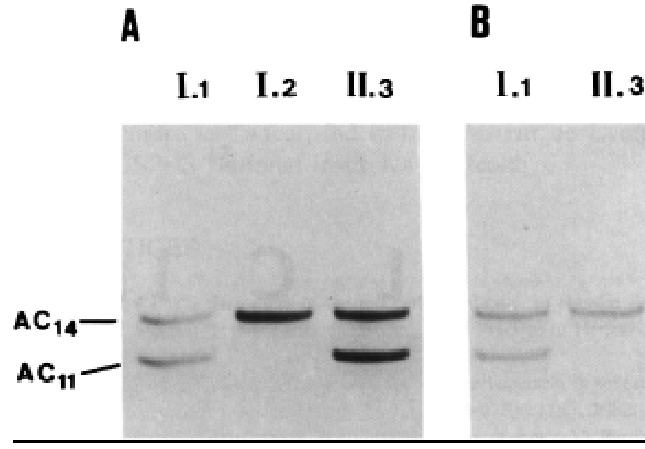


**Fig. 2. VNDR analysis at the genomic DNA (A) and cDNA (B) levels. Genomic and complementary cDNA were PCR-amplified using primers A and B (see text). The fragments obtained were separated using polyacrylamide gel electrophoresis and visualized with silver staining. Number of AC repeats (11 or 14) is indicated.**

## RESULTS

### Protein Analysis

In the unsplenectomized child II.3, the profile of ankyrin bands was modified due to the high reticulocyte count [30–32]. The amount of band 2.1 appeared normal (Table II; Fig. 1). Band 2.2, which is the product of a spliceoform of the *ANK1* transcript [33,34] (reviewed in Lux and Palek [1] and Peters and Lux [35]), also appeared normal. The ratio of ankyrin bands (2.1 ± 2.2) to band 3, expressed as a percentage, remained within normal limits. Following splenectomy, band 2.1 was diminished (with a concomitant lower reticulocyte count), and band 2.2 was also reduced. The amount of ankyrin bands (2.1 ± 2.2) was reduced by 33%. The spectrin and protein 4.2 bands were slightly but significantly decreased irrespective of splenectomy status (Table II). The protein patterns were normal for the parents and all other family members (not shown). In sum, the results of protein analysis in child II.3 strongly suggested that the *ANK1* gene carried the mutation.

### Analysis of *Nco*I Polymorphism and Variable Number of Dinucleotide Repeats (VNDR)

*Nco*I polymorphism (Table III) and VNDR analysis (Table III; Fig. 2) at the gene level excluded homozygosity of *ANK1* alleles in child II.3. Given the VNDR genotype of child II.3 ($AC_{14}/AC_{11}$), it was significant that only the $AC_{14}$ allele was detectable at the cDNA level. It looked as if one of the alleles ($AC_{11}$, of paternal origin) had been inactivated. We assumed that this allele carried a de novo mutation, resulting in the absence of the corresponding mRNA.

### SSCP Analysis of Ankyrin Gene Exons

We screened *ANK1* gene exons in the father, the mother, and child II.3, using SSCP analysis. Following PCR amplification, the fragments corresponding to exons

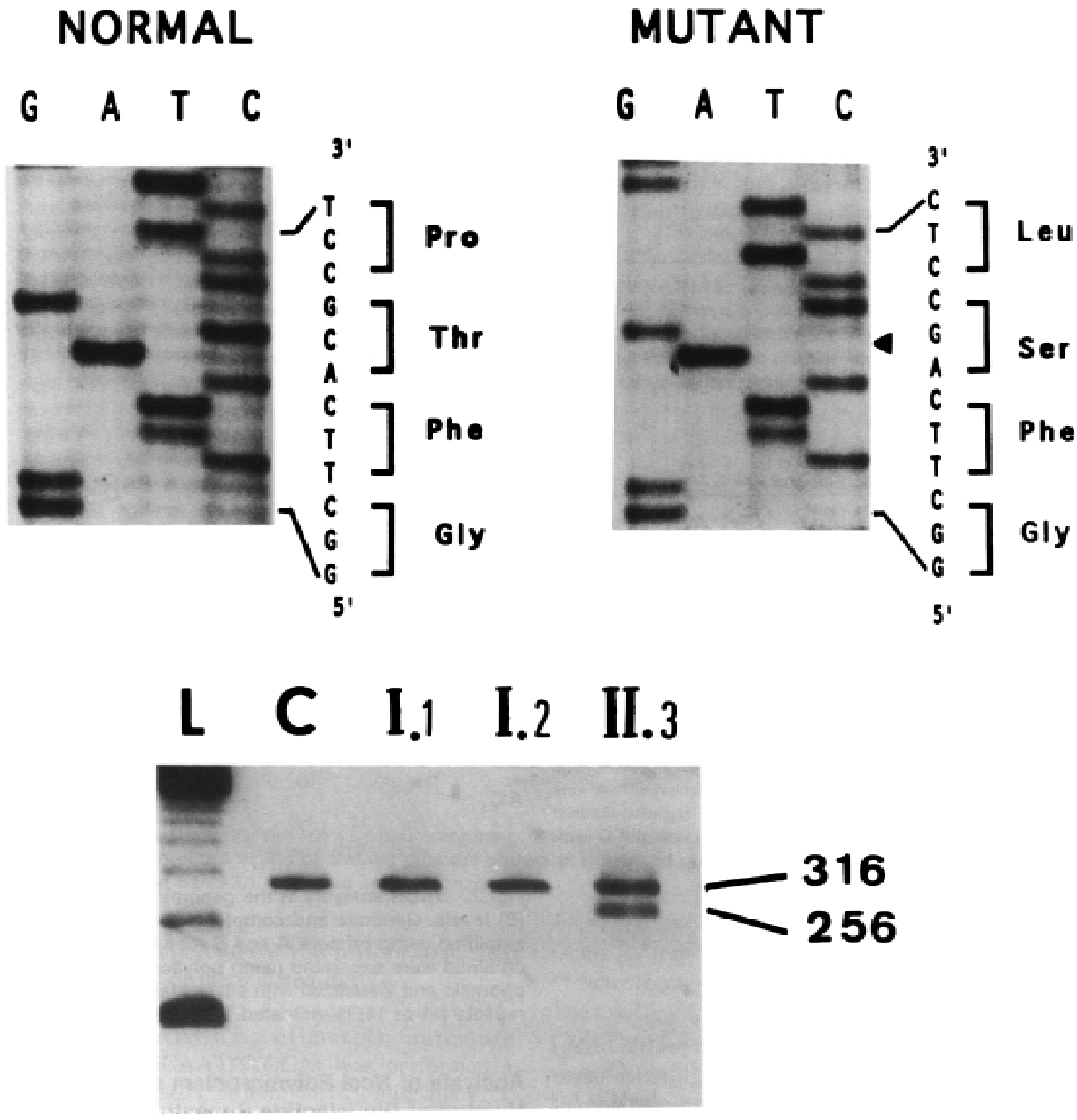


**Fig. 3. Nucleotide sequencing of normal and mutant exon 6 PCR products in child II.3 (top), and restriction analysis of PCR-amplified genomic DNA (bottom). Top: →, C, deletion. Bottom: The mutation creates an *Alw*NI site in exon 6 of the $ANK_1$ gene. This site is present in genomic DNA of child II.3 (256 nt), and absent in the father (I.1), the mother (I.2), and a control (C) (316 nt).**

1–5 appeared normal. The fragment (316 nt) carrying exon 6 showed an abnormal conformation in child II.3, but not in the parents (not shown). This result further supported the possibility of a de novo event and prompted us to clone and sequence exon 6 of child II.3.

### Nucleotide Sequencing and Restriction Analysis

Nucleotide sequencing of the cloned fragment carrying exon 6 disclosed the deletion of C (nt 521) in child II.3 (Fig. 3). This deletion gave rise to a missense amino-acid sequence starting from codon 146 and ending with a TAG triplet (that in nonmutated DNA overlaps codons 150 and 151: GTA GCC).

The mutated allele was designated ankyrin Bugey. At the gene level, one base deletion (−C) created an *Alw*NI restriction site. This site was present in the genomic DNA of the proposita, but not in the father or mother (Fig. 3). The deletion, therefore, appeared as a de novo mutation in child II.3, given the very high likelihood of paternity (see Case Report).

In child II.3, *Alw*NI digestion of the cDNA fragments (exons 3–7) showed that the mutated cDNA was not detectable (not shown). A trace amount of mutated cDNA was detected when larger cDNA fragments (exons 3–13) were amplified. No abnormally spliced products, such as one missing exon 6, were found (not shown). The patient's cDNA was almost entirely derived from the maternal ankyrin gene.

## DISCUSSION

In ankyrin-related HS patients, the extent of the reduction in the ankyrin content may be different from one patient to another [9–12,17]. The decrease in band 2.1 may be masked by a high reticulocyte count [30–32] in unsplenectomized HS patients. A reduction in the amount of spectrin and protein 4.2 is also usually observed but is smaller than the ankyrin primary deficiency [7,9,12,36]. In this report, the amount of spectrin was only slightly lowered, although the reduction may have been underevaluated. SDS-PAGE quantitation appears to lead to an overestimation of the spectrin content when compared with a radioimmunoassay method [9,37]. Following splenectomy, there was a clear-cut reduction in the amount of band 2.1. In unsplenectomized HS patients with a normal *ANK1* gene, the amount of band 2.1 is also increased [31,32] (also Alloisio et al., unpublished results) due to the higher reticulocyte count, but it returns to the normal range following splenectomy.

Eber et al. [18] described the first *ANK1* gene mutations associated with HS in a number of different German families. These mutations exhibited great heterogeneity with regard to their location. Mutation Bugey is located in repeat 4 of the 89-kDa domain carrying the binding site for band 3.

It is highly likely that allele Bugey will be transmitted in a dominant fashion and will be similarly expressed in future generations. Of course, we cannot rule out the possibility of a mutation in the proband, such as a missense mutation which remains unexpressed in the simple heterozygous state and which occurs in the *ANK1* allele in *trans.* This would enhance the expression of inactivated mutation Bugey. This possibility, however, is unlikely because the proband displays a clinical picture comparable to that of patients with dominantly-transmitted HS associated with a variety of nonsense mutations in one *ANK1* allele or with the deletion of one *ANK1* allele [7].

Allele Bugey is associated with trace amounts of the corresponding mRNA. It has been repeatedly observed that when the translation of mRNA is prematurely terminated, the mRNA is undetectable or at a very low level [38–42]. It has been reported that nonsense mutations in an exon can result in the skipping of this exon [42,43]. We have eliminated the possibility that the nonsense mutation caused exon 6 to be skipped.

In this report, we described a de novo mutated and novel variant of ankyrin. Previously, some de novo mutations of the *ANK1* gene were recorded in some HS cases [18,19]. The *ANK1* gene might be more prone to de novo alterations than many other genes.

## ACKNOWLEDGMENTS

We thank family NI for their cooperation and Mrs. N. Connon for careful preparation of the manuscript. This work was supported in part by the Central National de la Recherche Scientifique (CNRS URA 1171), the Programme Hospitalier de Recherche Clinique (1995–1997), the Université Claude-Bernard Lyon-I, the Institut National de la Santé et de la Recherche Médicale (CRE 930405), the Conseil de la Région Rhône-Alpes, the Fondation pour la Recherche Médicale, the Association Française contre les Myopathies, the Association de Recherche contre le Cancer, the Institut Pasteur de Lyon, and the USPHS National Institutes of Health.

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